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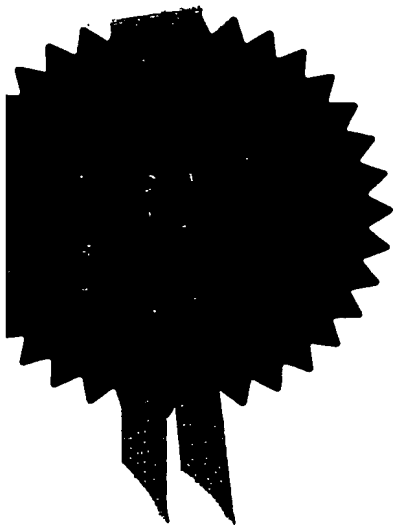

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2.	Patent application number *(The Patent Office will fill in this part)*	**0210535.1** — **8 MAY 2002**
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	**NOVARTIS AG** **LICHTSTRASSE 35** **4056 BASEL** **SWITZERLAND**
	Patent ADP number *(if you know it)*	7125497005
	If the applicant is a corporate body, give the country/state of its incorporation	**SWITZERLAND**
4.	Title of invention	**Organic compounds**
5.	Name of your agent *(If you have one)*	
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**B.A. YORKE & CO.** **CHARTERED PATENT AGENTS** **COOMB HOUSE, 7 ST. JOHN'S ROAD** **ISLEWORTH** **MIDDLESEX TW7 6NH**
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Organic Compounds

The present invention relates to organic compounds, e.g. to a method for detecting multiple analytes in a medium.

It is often desirable to determine the presence and the amount of a specific material in solution in a medium. Assays for determination rely in general on the interaction of an analyte, i.e. the material to be assayed, with a recognition molecule, i.e. a complementary molecule that will preferentially bind to a corresponding partner analyte. Interaction between an analyte and the corresponding recognition molecule thus may result in the formation of an analyte bound to a recognition molecule, hereinafter designated as "complex". Formation and amount of a formed complex may be determined, e.g. according to a method as conventional, e.g. by labeling a recognition molecule with a detection element. Said labeling may e.g. include further reacting a complex obtained with a detection element, e.g. a detection element which specifically binds to an analyte in a complex without interfering with the binding of the analyte to the recognition molecule. A detection element includes e.g. enzyme or fluorescence labelled reagents.

Generally such assays may be run by contacting a medium containing an analyte with a recognition molecule, optionally further contacting a complex formed with a detection element and determining the amount of complex formed. Said contacts are generally performed in micro titer plate wells containing the analyte. An analyte and the extent (amount) of its expression may be detected by addition of a recognition molecule and optionally a detection element. E.g. in case that several analytes are desired to be determined a complicated procedure may be necessary.

We have now found that such complicated procedure may be facilitated.

In one aspect the present invention provides an assay comprising
- a medium comprising a cell with the ability to express an analyte on stimulation,
- means for stimulating said cell to express such analyte,
- optionally means for cell disruption,
- a matrix comprising pins which are coated with a specific recognition molecule, and
- means for detecting the amount of the complex formed on said pins.

A cell includes one or more cells or cell lines, preferably a cell line, with the ability to express an analyte upon stimulation in a medium. An analyte is the material to be assayed and includes one or more anlalytes which are soluble in the medium. Analytes may be expressed extra- or intracellularly by a cell (cell line) upon stimulation. Analytes as used herein include one or more polypeptides / oligopeptides / oligonucleotides which are capable of mediating in vivo events, such as cytokines, chemokines, receptors, antibodies and oligonucleotides. Cytokines are a class of compounds which regulate responses of cells of the immune system, such as B and T lymphocyte cells ("B cells" and "T cells"), natural killer ("NK") cells, antigen presenting ("APC") cells. A "cytokine" is a soluble substance released by certain cell populations on contact with an inducer (stimulant) and which acts as an intercellular mediator. The terms "cytokine" and "lymphokine" have become interchangeable. In an attempt to simplify the nomenclature of these compounds, a group of participants at the Second International Lymphokine Workshop held in 1979 proposed the term "interleukin," abbreviated "IL," to develop a uniform system of nomenclature based on the ability of the proteins to act as communication signals between different populations of leukocytes. Cytokines preferably include interleukines, preferably IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, interferones, preferably IFN-α, IFN-β, IFN-γ, the tumor necrosis factor (TNF molecules), e.g. TNF-α, TNF-β, the transforming growth factor TGF, including the TGF-β superfamily selected from the group of cytokines consisting of the TGF-β family, the inhibin family, the DPP/VG1 family, and the Mullerian Inhibiting Substance family, and the granulocyte-macrophage colony stimulating factor GM-CSF. Cytokines include cytokine receptors. "Cytokine (superfamily) receptors" are a group of closely related glycoprotein cell surface receptors that share considerable homology including frequently a WSXWS domain and are generally classified as members of the cytokine receptor superfamily. Members of the superfamily include, but are not limited to, receptors for: IL-2 (α, β and γ chains), IL-3, IL-4, IL-5; IL-6, IL-7, IL-9, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), Leukemia inhibitory factor (LIF); oncostatin M (OSM) and also receptors for prolactin, growth hormone (GH), ciliary neurotrophic factor (CNTF). These receptors are generally expressed as transmembrane proteins but they can also be found in solution in medium samples.

Chemokines, also known as "intercrines" and "SIS cytokines", comprise a family of small secreted proteins (e.g. 70-100 amino acids and about 8-10 kiloDaltons) which attract and

activate leukocytes and thereby aid in the stimulation and regulation of the immune system. The name "chemokine" is derived from chemotactic cytokine, and refers to the ability of these proteins to stimulate chemotaxis of leukocytes. Indeed, chemokines may comprise the main attractants for inflammatory cells into pathological tissues (see generally, Baggiolini et al., Advances in Immunology, 55:97-179 (1994)). Previously identified chemokines generally may exhibit 20-70% amino acid identity to each other and contain four highly-conserved cysteine residues. Based on the relative position of the first two of these cysteine residues, chemokines have been further classified into subfamilies. In the "C-X-C" or "α" subfamily, encoded by genes localized to human chromosome 4, the first two cysteines are separated by one amino acid. In the "C-C" or "β" subfamily, encoded by genes on human chromosome 17, the first two cysteines are adjacent. X-ray crystallography and NMR studies of several chemokines have indicated that, in each family, the first and third cysteines form a disulfide bridge, and the second and fourth cysteines form a second disulfide bridge, strongly influencing the native conformation of these proteins. Families of chemokine proteins are described in more detail in Zlotnik et al., 12 Immunity 121-27 (2000) and Saunders et al., 4 DDT 80-92 (1999).

A cognate receptor for a chemoattractant is a receptor that can interact with the chemoattractant molecule. Cognate, in general, refers to biomolecules that typically interact, for example, a receptor and its ligand. Chemokines include, but are not limited to, chemokine receptors and preferably include the chemokine receptors CXCR1, CXCR2, CXCR3, CXCR4, CXCR5,CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CCR11, XCR1, CX_3CR1, C5a receptor, arachidonate derivative leukotriene B_4 receptor, platelet activating factor receptors, formyl-met-leu-phe receptor, neutrophil activating protein-1 receptor, interleukin 8 receptor, platelet factor 4 receptor, platelet basic protein receptor, melanoma growth stimulating factor/GRO receptor.

Means for stimulating cells to express analytes as described herein are known, or may be found analogously to known methods, or are as described herein. Stimulation agents include e.g. antibodies, antigens, superantigens and chemical compounds. Means for cell disruption are known or may be found analogously to known methods. Cell disruption may be carried out in case that an analyte which is desired to be detected is expressed intracellularly.

A matrix comprising pins consists of a matrix wherein pins are located, herein also designated as "pin system". A matrix is preferably a plastic matrix. Pins are preferably plastic

pins or (pre-treated) glass pins, preferably plastic pins, with the ability to bind a recognition molecule, e.g. directly or via a linker molecule. Plastic or (pre-treated) glass material with the ability to bind (stably) a recognition molecule is known or may be provided analogously to a method as conventional. The pins conveniently should all have the same seize. The length of the pins should be such, that all pins can be dipped into wells (e.g. wells of micro-titer plates), each pin in a distinct well simultanously. A preferred pin length include 10 mm and more, e.g. 10 mm to 400 mm, preferably 20 to 300 mm, such as 40 to 200 mm. These pins should have an appropriate thickness which allows convenient coating of the pins and which allows convenient dipping of the pins into wells, e.g. 0.2 to 2.0 mm, such as 0.3 to 1.5 mm. The size of that matrix should be such, that it can be conveniently handled, either by hand or by a robot machine. The seize of that matrix is e.g. dependent on the number of pins which are desired and the well system were they are intended to be inserted. Preferably 96 and more pins, e.g. 96 to 100000 pins. A convenient number of pins include that number which fits into existent standard microtiter plates; e.g. 96, 384 or 1536, e.g. arranged in a format that fits existent standard microtiter plates; but miniaturization of pin sizes and matrix formats to accommodate standard oligonucleotide arrays is also an option. The pins are located such, that each pin is distinct from its neighboring pins. These pins are regularly located in the matrix, preferably such, that each pin can be dipped distinctly in one well simultanously. For that a well system should be provided with a number of wells which corresponds to the number of pins and wherein the wells are located such, that the pins of the pin system can be dipped into the well system simultanously, in a way that each pin may be dipped distinctly in one well.

These pins are coated with a recognition molecule. A recognition molecule includes such complementary molecules that will preferentially bind to a corresponding partner analyte, e.g. including antibodies, receptors, e.g. including fragments of antibodies, receptors; and other substrates; e.g. a cytokine, in case that a cytokine receptor is used as an analyte, or vice versa, e.g. a cytokine receptor, in case that a cytokine is used as an anyalyte, e.g. a oligonucleotide sequence, in case that an oligonucleotide containing a complementary sequence is used as an analyte. Pin coating may be effected analogously to a method as conventional, and is preferably simply effected by dipping the pins of the pin system in a well system, wherein the wells are filled with a recognition molecule in appropriate medium, to obtain a matrix comprising pins coated with a recognition molecule.

An analyte may be captured by (bound to) an analyte-specific recognition molecule when dipping the coated pins into wells containing such analyte, e.g. by dipping the pins into wells

containing stimulated intact cells or by dipping the pins into wells containing stimulated cells after disrupting their membranes to allow the release of intracellular analytes in the medium containing the cells; e.g. in the absence or in the presence of agonists or antagonists.

Means for detecting the amount of the complex formed on the (coated) pins include means for detecting the amount of a complex formed in normal assays, e.g. ELISA, DELFIA and oligonucleotide tagging assays. Preferably the analyte on the pins is detected by dipping the pin system in a well system wherein the wells are filled with an appropriate detection element, e.g. in appropriate medium. An appropriate detection medium includes e.g. a substrate medium containing a detection element that is capable to change optical or fluorescence properties in contact with the coating on the pins, including but not restricted to horseradish peroxidase substrates, alkaline phosphatase substrates, luciferase substrates, time resolve fluorescence substrates and enhancement solutions, and polymerase chain reaction solutions.

The assay according to the present invention can also be provided in the form of a kit. An (assay) kit will usually contain recognition molecule-coated pin system(s), corresponding well system(s) and detection element(s), e.g. and may further comprise one or more of the following: a calibration standard for desired analyte(s) (calibration sample(s)), control sample(s) containing known amounts/concentrations of the desired analyte(s), and instructions for using the components of said kit to quantify or simply to detect the analyte(s) in a sample.

An assay (kit) according to the present invention may be used for the detection of one or more analytes in a medium which are expressed by a cell (line) upon stimulation, or, for determination, whether an analyte is expressed in a lower amount or in a higher amount, or is not expressed at all, if a candidate compound is present.

In another aspect the present invention provides a kit comprising

a. recognition molecule-coated pin system(s),
b. corresponding well system(s),
c. detection element(s), and optionally
d. a calibration standard for desired analyte(s) (calibration sample(s)),
e. control sample(s) containing known amounts/concentrations of the desired analyte(s),

f. instructions for using the components of said kit to quantify or simply to detect the analyte(s) in a sample.

In another aspect the present invention provides a process for the detection whether, and optionally to what amount, one or more analytes are expressed in a medium, comprising the steps

a. stimulating a cell with the ability to express an analyte on stimulation in appropriate medium,
b. contacting said medium after stimulation with pins of a matrix comprising pins which are coated with a specific recognition molecule, optionally after cell disruption,
c. optionally, e.g. preferably, contacting coated pins obtained in step b. with a detection element, and
d. determining whether, and, optionally, e.g. preferably, to what extent, the analyte has been expressed in step a..

In step b. the medium otained from step a. is preferably incubated in the presence of coated pins for a period of time which is sufficient for complex formation. A necessary time period may be determined by pre-testing. Contacting in step b. is preferably carried out by dipping the pins of the pin system obtained in step b. in a corresponding well system wherein the wells are filled with a medium comprising an analyte-specific recognition molecule. Cell disruption in step b. may be carried out if the analyte to be determined is intracellularly expressed before carrying out step b.. Contacting coated pins in step c. with a detection element may be carried out by dipping the pin system obtained in step b. into a corresponding well system wherein the wells are filled with appropriate substrate medium containing a detection element. Determination according to step d. is carried out as appropriate, e.g. depending on the detection element used, e.g. including (fluorescence) measurement at appropriate wavelengths, or appropriate enzyme kinetic measurement.

A method according to the present invention may be advantageous in comparison with prior art processes, because one well comprising a stimulated cell with the ability to express an analyte on stimulation in appropriate medium may be contacted several times with a matrix comprising pins which are coated, each with a specific, but different recognition molecule, which different recognition molecules are specific for different analytes expressed. Thus only one single well is in principle necessary to determine several different expressed analytes.

Also, several analytes may be detected simultaneously in one single detection procedure side by side. Washing procedures between distinct determination steps, such as between contacting with recognition molecule and contacting with a detection element, e.g. as necessary in ELISA detection procedures, may be avoided.

A method according to the present invention may be used for identifying agonists or antagonists of the expression of analytes.

In another aspect the present invention provides a process for the identification of (ant)agonists of the expression of analytes by a cell, comprising the steps

a. stimulating a cell with the ability to express analyte(s) on stimulation with a stimulant which results in analyte(s) expression of said cell in a medium in the absence of a candidate compound,
b. stimulating a cell with the ability to express analyte(s) on stimulation with a stimulant under the conditions of step a. in the presence of a candidate compound,
c. contacting the medium of step a. and the medium of step b. after stimulation each with a matrix comprising pins which are coated with a specific recognition molecule, e.g. at least one time, or several times, each time with recognition molecule(s) which are specific for one or more expressed analyte(s), e.g. optionally after cell disruption, and
d. determining whether and to which amount an analyte in the presence of a candidate compound is expressed by comparison with analyte expression in step a..

Agonists and antagonists may e.g. include (poly)peptides, monoclonal antibodies, low molecular weight chemical compounds, antisense oligonucleotides. (Ant)agonists identified according to the present invention may be useful as pharmaceutically active compounds by interfering in the production of an anlyte in vivo. A candidate compound include low molecular weight compounds, e.g. in chemical libraries and natural product libraries, and antisense oligonucleotides, e.g. present in natural or synthetic compound libraries, i.e. systematic collections of chemical entities, for which the affect on the production of an analyte of a cell in step b. is unknown.

In another aspect the present invention provides a process for the identification of pharmaceutically active compounds, e.g. and for using (ant)agonists detected, comprising the steps

a. stimulating a cell with the ability to express analyte(s) on stimulation with a stimulans which results in analyte(s) expression of said cell in a medium in the absence of a candidate compound,

b. stimulating a cell with the ability to express analyte(s) on stimulation with a stimulans under the consitions of step a. in the presence of a candidate compound,

c. contacting the medium of step a. and the medium of step b. after stimulation each with a matrix comprising pins which are coated, each with a specific recognition molecule, e.g. at least one time, or several times, each time with recognition molecule(s) which are specific for one or more expressed analyte(s),.

d. determining whether and to which amount an analyte in the presence of a candidate compound is expressed by comparison with analyte expression in step a., and

e. choosing, e.g. and using, an (ant)agonist identified as a pharmaceutically active compound.

A specific pharmaceutical activity of an (ant)agonist is dependent on the nature of the analyte(s) detected and includes pharmaceutial activity in diseases which are mediated by the release of one or more specific analyte(s) in vivo, e.g. wherein analyte(s) are selected from one or more proteins which are capable of mediating in vivo events, such as cytokines, chemokines, receptors, antibodies and oligonucleotides.

In another aspect the present invention provides an assay, a kit and a process for the identification of (ant)agonists of the expression of inhibitors of analytes, wherein the analytes are selected from the group consisting of human IL-4 and/or IL-10 and/or IFN-γ.

In another aspect the present invention provides an assay, a kit and a process for the identification of (ant)agonists of the expression of inhibitors of analytes, wherein the recognition molecules are selected from the group consisting of mAbs of human IL-4 and/or IL-10 and/or IFN-γ.

In another aspect the present invention provides a process for the detection whether, and optionally to what amount, one or more analytes are expressed in a medium wherein the detection element is selected from the group consisting of horseradish peroxidase substrates, alkaline phosphatase substrates, luciferase substrates, time resolve

fluorescence substrates and enhancement solutions, and polymerase chain reaction solutions, preferably time resolve fluorescence substrates and enhancement solutions.

$CD4^+$ T helper cells (Th) play a major role in regulating the effector mechanisms that the adaptive immune system has developed to combat different pathogens. Th cells recognize specific antigen peptides, presented by professional antigen presenting cells (APC), in the context of Major Histocompatibility Class II (MHC-II) molecules. Upon activation, Th cells proliferate and differentiate into discrete Th subsets defined on the basis of their cytokine secretion patterns. Th cell differentiation is a multifactorial decision determined by factors such as the genetic background, the dose of antigen, the density of costimulatory molecules and, above all, the cytokine milieu in which the immune response takes place (see e.g. Coffman RL and Reiner SL, Science (1999), 284: 1283). Thus, IL-12 is responsible for driving Th cells into the Th1 phenotype, which is characterized by the production of IL-2, IFN-γ and TNF-β. Th1 cells are involved in pro-inflammatory responses that are characteristic of cell-mediated immunity and therefore they are important for the clearance of many infectious organisms. Th2 cells produce IL-4, IL-5 and IL-13 and their development is induced by IL-4. Th2 cells are predominantly helper of antibody-mediated B cell responses and exert anti-inflammatory functions. Generally, naïve $CD45RA^+$ Th cells first progress to a Th0 phenotype, a subset that is capable of producing both Th1 and Th2 cytokines. Following repeated stimulation with specific antigens, Th0 cells differentiate further into Th1 and Th2 cells. The resulting Th1 or Th2 cell subsets remain predominant throughout a particular immune episode in part because of counter-inhibitory effects of cytokines secreted by the reciprocal subset (see e.g. O'Garra A. Immunity (1998), 8: 275). However, exacerbated Th1 or Th2 responses might be detrimental for the host. Hence, Th1 cells are implicated in the immunopathology associated with autoimmune diseases whereas Th2 cells mediate allergic disorders. Fortunately, most human Th cells co-express various combinations of Th1 and Th2 cytokines. These Th0 cells may also represent stable subsets with balanced Th1/Th2 cytokine production that are functionally involved in the clearance of pathogens while inducing minimal immunopathology. In addition, $CD4^+$ T cells may also differentiate, in the presence of IL-10, into T regulatory (Tr1) cells. Tr1 cells produce high levels of IL-10 and TGF-β1 and exert downregulatory effects on both Th1 and Th2 responses(see e.g. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, De Vries JE and Roncarolo MG, Nature (1997), 389: 737).

It is evident that the immune system has evolved to generate the type of immune response most appropriate for each particular situation and that, in addition, it has developed control mechanisms to switch off these responses once the danger signals are cleared. However, despite of these regulatory mechanisms, situations of extreme Th polarization resulting in immunopathology often occur and then, it would be extremely beneficial to have a suitable active substance capable of antagonizing the effector functions of the polarizing cytokine and/or reverting the ongoing immune response into a non-pathological outcome. According to the present invention we have found the basis of a high throughput screening (HTS) assay to identify modulators of human IL-4, IFN-γ and IL-10 production. These cytokines play important roles in the processes of differentiation and/or effector functions of Th cells, including immunopathological situations. Antagonists, e.g. specific antagonists, of IL-4 production would be candidates to target allergic diseases, antagonists, e.g. specific antagonists, supressing IFN-γ production may be beneficial for the treatment of autoimmune diseases, and agonists, e.g. specific agonists, of IL-10 production would be potential downregulators of immune responses and are useful in the treatment and prophylaxis of corresponding diseases.

The present invention provides a facilitated screening method (identification method for (ant)agonists) in comparison with previous screening processes, e.g. in that one single well system wherein cell stimulation is carried out may be used for determination of a series of different analytes, e.g determining the complex formation on the pins of a pin system several times, e.g. using at each time a pin system wherein the pins are coated differently, e.g. each time specificly coated for complex formation with desired analyte(s); e.g. and in that washing of titer plates used according to previous procedures may be avoided. Thus, a method according to the present invention may be appropriate for quick and efficient high throughput screening (HTS).

Description of the Figures

Figure 1 shows cytokine production of #98016T0 cells. T cell stimulation was performed as described under Preparation of a cell line which produces analyte-containing medium above, at day 12 after re-stimulation. Intracytoplasmic staining was performed after 4 hours of activation. The amount of cytokine production measured by ELISA after 24 hours of activation was 5.2 ng/ml of IL-2, 13.7 ng/ml of IFN-γ, 7.9 ng/ml of IL-4, 0.1 ng/ml of IL-5 and 24,6 ng/ml of IL-10.

Figure 2 shows the detection of recombinant human cytokine standards following the DELFIA procedure. Recombinant human IL-4 (R&D Systems, Minneapolis, Minnesota), IL-10 (PharMingen) and IFN-γ (R&D Systems) were used as standards. Washing and blocking solutions were the same as for the ELISA method as described above. All samples were assayed in triplicate. Each point represents the average result ± standard deviation. All 3 cytokiners were detected in a mix solution using tissue culture supernatant as diluent. These results indicate that the triple-DELFIA detects <50 pg/ml of each cytokine.

Figure 3 shows results obtained using T-cell supernatants of serial dilutions of stimulated #98016To cells. These results were obtained using anti-CD3 immunobeads plus anti-CD28 mAb as stimulatory signals. Both, T cell stimulation and DELFIA assays were carried out using 384-well plates as described above. All samples were assayed in triplicate. Each point represents the average result ± standard deviation.

Figure 4 illustrates a characteristic dose dependent inhibition of IFN-γ, IL-4 and IL-10 production of #98016T0 cells by CsA in an assay according to the present invention. T cells were activated for 24h with anti-CD3 immunobeads and anti-CD28. All samples were assayed in triplicate. Each point represents the average result ± standard deviation.

EXAMPLES

Example 1

Preparation of a cell line which produces analyte-containing medium

IL-4, IFN-γ and IL-10 are chosen as analytes according to a preferred embodiment of the present invention.

A human $CD4^+$ Th_0 cell line, i.e. cell line #98016T_0, from a skin biopsy of a patient with atopic dermatitis (AD) is isolated according to the moethod as described in Carballido JM, Aversa G, Kaltoft K, Cocks BG, Punnonen J, Yssel H, Thestrup-Pedersen K and de Vries JE. J Immunol (1997), 159: 4316. Briefly, patients with AD, who are allergic to Der p 1 as judged by specific cutaneous prick test and serum IgE levels are patch challenged with Der p 1. Punch biopsies (4 mm) are obtained from the patients that underwent a Delayed Type Hypersensitivity reaction after 24 hours exposure to the allergen, cut in four approximately equally sized pieces and cultured at 37°C in a humidified atmosphere under 8% CO_2 in 12-well plates (Costar, Cambridge, MA) in Yssel's medium (Gibco BRL) containing 1% human AB serum and 1 µg/ml of purified Der p 1 (ALK Laboratories, Hørsholm, Denmark). The cultures are supplemented with 100 U/ml of rhIL-2 and 400 U/ml of rhIL-4 in the presence and in the absence of 0.3 ng/ml of rhIL-12 (R&D Systems, Minneapolis, MN). Th cells migrated out of the skin biopsies and proliferated with comparable doubling times under the different culture conditions. Culture medium containing the relevant cytokines is replaced every 2 to 3 days. After 14 days of expansion, the cell cultures are re-stimulated with 1 µg/ml of purified Der p 1 and re-expanded in medium supplemented with the cytokine cocktails. At the end of the second culture cycle, $>5 \times 10^7$ cells are recovered from each condition. Subsequently, human Th cell lines are maintained in Yssel's medium 10% FCS by repeated stimulation using 1 µg/ml PHA and allogeneic (50 Gy irradiated) peripheral blood mononuclear cells, as a source of feeder cells, followed by expansion on IL-2 and IL-4 ± IL-12. The $CD4^+$ cell line #98016T_0 is selected because these cells, upon stimulation, consistently displayed a Th_0 phenotype as judged by intracytoplasmic cytokine staining detected at the single cell level by FACS or by cytokine production at the total population level measured by ELISA (see e.g. also Figure 1).

FACS and ELISA (proof of cytokine production by the selected cell line)

Human #98016T_0 cells are harvested 12 to 16 days after re-stimulation, washed three times in PBS and stimulated in 24-well plates (Costar) using Yssel's medium containing 10% FCS, 5 µg/ml of the anti-CD3 mAb SPV-T3(5) and 1 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) (SIGMA, St. Louis, MO). The intracytoplasmic content of IL-4 and IFN-γ in

single cells is determined according to the method as described in Carballido JM, Aversa G, Kaltoft K, Cocks BG, Punnonen J, Yssel H, Thestrup-Pedersen K and de Vries JE. J Immunol (1997), 159: 4316. Briefly, the Th cells are treated with 10 µg/ml of Brefeldin-A (Epicentre Technologies, Madison, WI) 2 hours after stimulation and cultured for additional 2 hours. Next, the Th cells are harvested, fixed in 2% formaldehyde, permeabilized with saponin and stained with anti-IL-4 and anti-IFN-γ mAb conjugated to PE and FITC, respectively (PharMingen, San Diego, CA). The Th cells are analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA). To determine total cytokine production the Th cells are harvested and stimulated for 24 h at 10^6 cells/well using 24-well plates (Costar) in 1 ml of Yssel's medium containing 10% FCS, 5 µg/ml of the anti-CD3 mAb SPV-T3 and 1 ng/ml of TPA. Cytokine production is measured by specific sandwich ELISA as follows: Maxisorp C96-well immuno-plates (NUNC, Roskilde, Denmark) are coated overnight at room temperature (RT) with 50 µl/well of 3 µg/ml solution of specific capturing mAb in Carbonate buffer (SIGMA C-3041). After coating, the plates are washed with PBS-0.05% Tween 20 and blocked at RT by 1 hour incubation with 200 µl of casein hydrolysate (OXOID L41) and 5% Tween 20 in PBS. Thereafter, the plates are incubated with 25 µ/well of the different T cell supernatants or specific standard plus 25 µl/well of biotinylated mAb. After overnight incubation at RT the plates are washed again and incubated for 2 hours with extravidin alkaline phosphatase conjugated (SIGMA E-2636). Read out is performed using p-Nitrophenyl phosphate (SIGMA N-2640) in 1M diethanolamine buffer as a substrate and measuring absorbance at 405-492 nm in a SpectraMAX 340 spectrophotometer (Molecular Devices, Sunnyvale, California) equipped with SoftMax software (Molecular Devices). The mAbs used for capturing and detection, respectively, are 8F12 and 3H-4 for the IL-4 ELISA and 43-11 and 45-15 for the IFN-γ ELISA (please see below). Production amounts of some cytokines are shown in Figure 1.

Example 2

HTS assay for human cytokine inhibitors

Step 1. T cell stimulation

Anti-CD3 mAb SPV-T3 is coupled to 100 mg of activated immunobead matrix (Irvine Scientific, Santa Ana, California) according to the manufacturer's instructions. The anti-CD3 coupled immunobeads are titrated in T-cell proliferation assays and used in all successive experiments at the final concentration of 75 µg/ml (equivalent to 2 µg/ml of anti-CD3 mAb). Anti-CD28 mAb (purchased from PharMingen, San Diego, California) is used at 5 µg/ml.

Serial dilutions of #98016T_0 cells are stimulated for 24 hours with anti-CD3 immunobeads and anti-CD28 mAb in Yssel's medium in the presence or absence of a candidate compounds. T-cell stimulation is performed in 40 µl volume using 384-well tissue culture plates (Nunc #164688).

Step 2. Pin coating with recognition molecules and complex formation

As recognition molecules according to the present invention mAbs against IL-4, IL-10 and IFN-γ are used, available according to a method as conventional. Coating mAbs for IL-4, IL-10 and IFN-γ DELFIA are designated herein as 8F12, JES3-12G8 and 43-11, respectively. Cytokine production is determined by time-resolved fluorescence in a Wallac 1420 Victor2 multilabel counter.

For this purpose, the pins of a matrix wherein 384 pins are located, which pins fit into a 384-well tissue culture plate (Nunc #164688) are coated by dipping these pins for 2 h at room temperature into 30 µl/well of a cocktail containing 2 µg/ml of mAb 8F12, 2.5 µg/ml of mAb JES3-12G8 and 2.5 µg/ml of mAb 43-11 in carbonate buffer, pH 9.6 (SIGMA C3041) and blocking for 1 hour at room temperature.

Step 3. Contact with the detection element and determination of analyte content

For detection of complex formation on the pins Enhanced Lanthanide FluoroImmuno Assay (DELFIA) is used.

As detection elements according to the present invention mAbs for IL-4, IL-10 and IFN-γ DELFIA are used which do not compete with the recognition molecule mAbs described in Step 2 above, designated as 3H4 (for IL-4), JES3-9D7 (for IL-10) and 45-15 (for IFN-γ), respectively. The 3H4 mAb is labeled with Samarium (Sm), the JES3-9D7 mAb is labeled with Terbium (Tb) and the 45-15 mAb is labeled with Europium (Eu). Lanthanide labeled mAbs may be obtained by Advant-Wallac, Turku, Finland. Recombinant human IL-4 (R&D Systems, Minneapolis, Minnesota), IL-10 (PharMingen) and IFN-γ (R&D Systems) are used as standards. Washing and blocking solutions are the same as for the ELISA method described above. DELFIA assay buffer #1244-111, enhancement solution #1244-105 and enhancer #C500-100 may be obtained from Wallac.

The coating of the pins of the matrix obtained in step 2 after incubation is incubated for 2 hours by dipping these pins into a 384-well tissue culture plate (Nunc #164688), each well being filled with 30 µl/well of a solution containing the three lanthanide-labeled mAbs (each, at [0.5 µg/ml] final) in DELFIA assay buffer, followed by dipping these pins into a 384-well tissue culture plate (Nunc #164688), each well being filled with 50 µl/well of Wallac Enhancement Solution. The 384-well tissue culture plate (Nunc #164688) obtained is

transferred to the reader for time resolved fluorescence determination in the Eu and Sm windows and the Eu (IFN-γ)– and Sm (IL-4)-content is determined. Thereafter, each well receives 15 µl of Wallac Enhancer and the plates are read in the Tb window to determine the Tb (IL-10)-content.

Example 3

Inhibition of cytokine production by a known cytokine-production inhibitor determined in an HTS assay for human cytokine inhibitors

According to the methods as described in Example 2, Steps 1 to 3 experiments using Cyclosporin A (CsA) as a prototype inhibitor of cytokine production as a proof of principle are carried out. For that, T-cell stimulation according to Example 2, Step 1 is carried out in the presence of different amounts of CsA and the other Steps and determination of cytokine production are carried out according to Example 2, Step 2 and Step 3. Results are shown in Figure 4 which illustrates a characteristic dose dependent inhibition of IFN-γ, IL-4 and IL-10 production of #98016T0 cells by CsA. This illustrates also the proof of principle of the assay and methods according to the present invention.

Patent claims

1. An assay comprising
   - a medium comprising a cell with the ability to express an analyte on stimulation,
   - means for stimulating said cell to express such analyte,
   - optionally means for cell disruption,
   - a matrix comprising pins which are coated with a specific recognition molecule, and
   - means for detecting the amount of the complex formed on said pins.

2. A process for the detection whether, and optionally to what amount, one or more analytes are expressed in a medium, comprising the steps
   a. stimulating a cell with the ability to express an analyte on stimulation in appropriate medium,
   b. contacting said medium after stimulation with pins of a matrix comprising pins which are coated with a specific recognition molecule, optionally after cell disruption,
   c. optionally contacting coated pins obtained in step b. with a detection element, and
   d. determining whether, and, optionally, to what extent, the analyte has been expressed in step a..

3. A process for the identification of (ant)agonists of the expression of analytes by a cell, comprising the steps
   a. stimulating a cell with the ability to express analyte(s) on stimulation with a stimulant which results in analyte(s) expression of said cell in a medium in the absence of a candidate compound,
   b. stimulating a cell with the ability to express analyte(s) on stimulation with a stimulant under the conditions of step a. in the presence of a candidate compound,
   c. contacting the medium of step a. and the medium of step b. after stimulation each with a matrix comprising pins which are coated with a specific recognition molecule, e.g. at least one time, or several times, each time with recognition molecule(s) which are specific for one or more expressed analyte(s), e.g. optionally after cell disruption, and
   d. determining whether and to which amount an analyte in the presence of a candidate compound is expressed by comparison with analyte expression in step a..

4. A process for the identification of pharmaceutically active compounds comprising the steps as claimed in claim 3 and further comprising step
e. choosing an (ant)agonist identified as a pharmaceutically active compound.

5. A process as claimed in claim 4 and further comprising step
f. using an (ant)agonist identified as a pharmaceutically active compound.

6. A kit comprising
a. recognition molecule-coated pin system(s),
b. corresponding well system(s),
c. detection element(s), and optionally
d. a calibration standard for desired analyte(s) (calibration sample(s)),
e. control sample(s) containing known amounts/concentrations of the desired analyte(s),
f. instructions for using the components of said kit to quantify or simply to detect the analyte(s) in a sample.

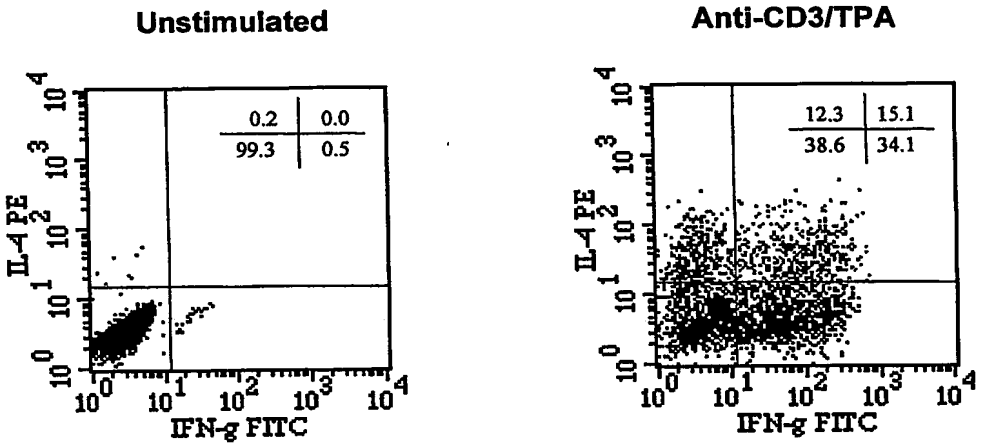
7. An assay, a kit or a process according to any one of the preceding claims, wherein the analyte is selected from the group consisting of human IL-4 and/or IL-10 and/or IFN-γ.

8. An assay, a kit or a process according to claim 7, wherein the recognition molecule is selected from the group consisting of mAbs of human IL-4 and/or IL-10 and/or IFN-γ.

Abstract

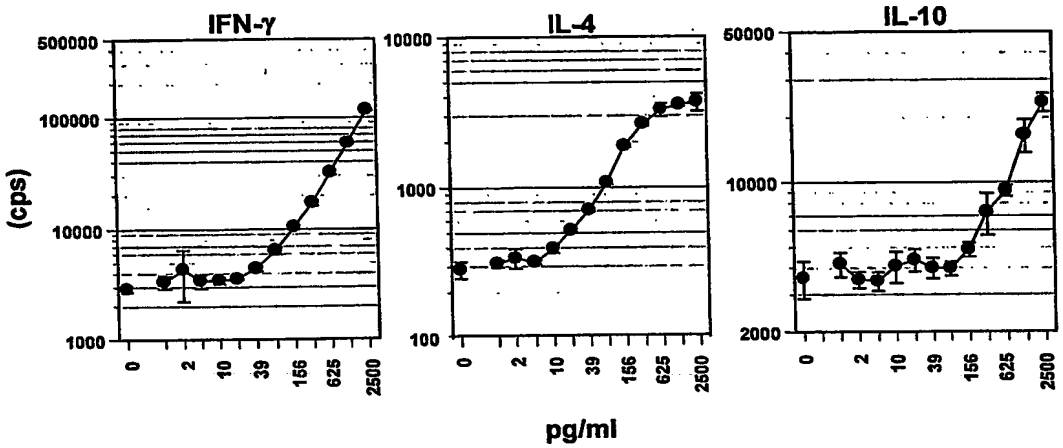
An assay comprising a medium comprising a cell with the ability to express an analyte on stimulation, means for stimulating said cell to express such analyte, optionally means for cell disruption, a matrix comprising pins which are coated with a specific recognition molecule, and means for detecting the amount of the complex formed on said pins, and use of such assay.

Figure 1/4



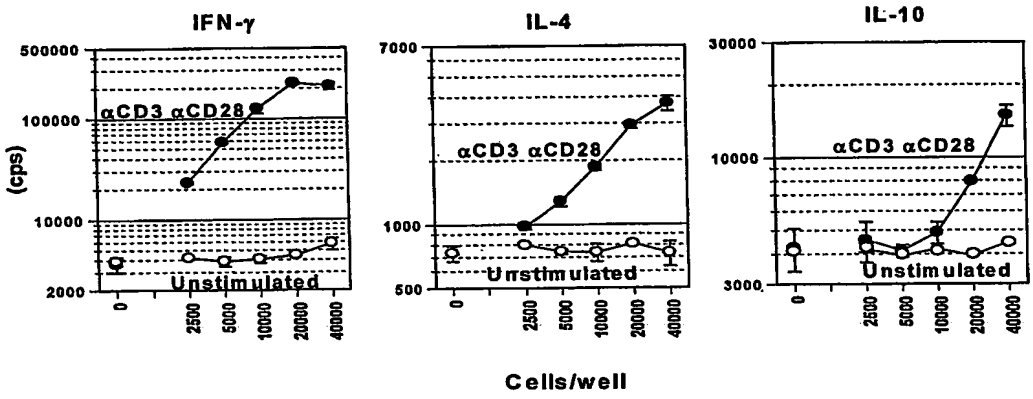
Cytokine profile of #98016T_0 cells

Figure 2/4



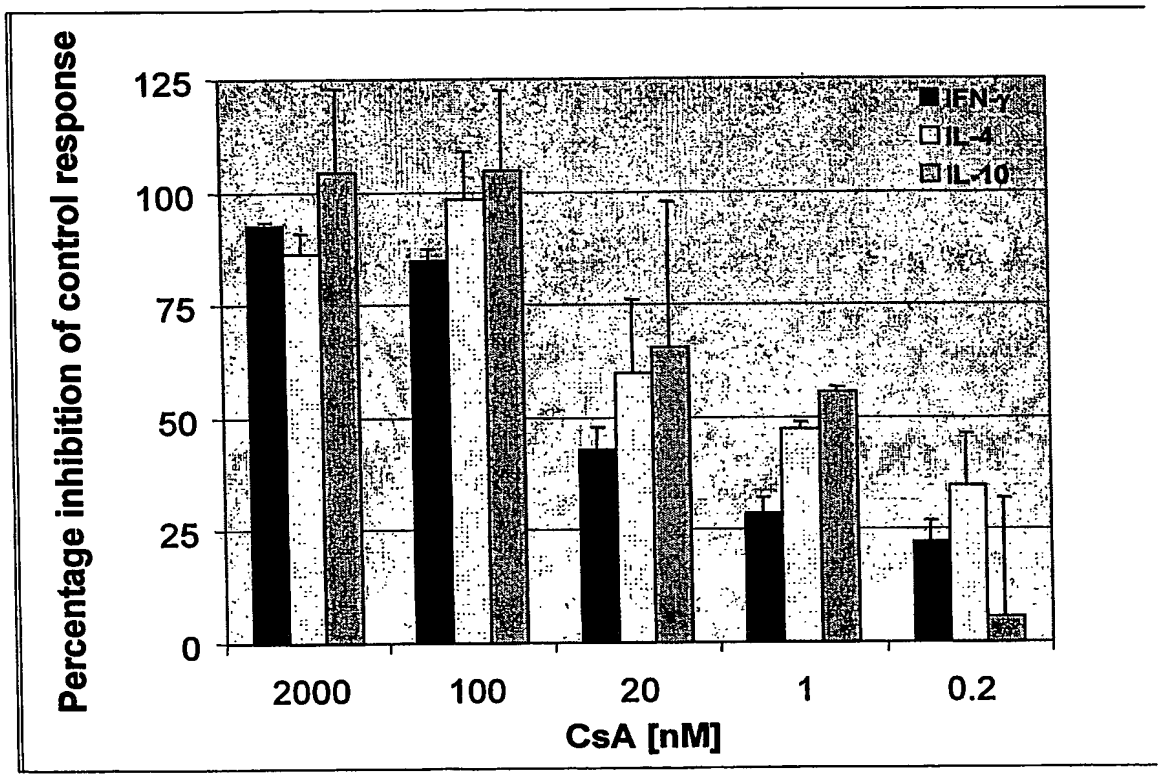
Triple cytokine DELFIA

Figure 3/4



Cytokine production by #98016T_0 cells

Figure 4/4



Suppression of human cytokine production by CsA